

Enzymatic Synthesis of Cytidine 5'-Monophospho-*N*-acetylneuraminic Acid

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We have established an efficient method for enzymatic production of cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) from inexpensive materials, *N*-acetylglucosamine (GlcNAc) and cytidine 5'-monophosphate (CMP). The *Haemophilus influenzae nanE* gene encoding GlcNAc 6-phosphate (GlcNAc 6-P) 2-epimerase and the *Campylobacter jejuni neuB1* gene encoding *N*-acetylneuraminic acid (NeuAc) synthetase, both of whose products are involved in NeuAc biosynthesis, were cloned and co-expressed in *Escherichia coli* cells. We examined the synthesis of NeuAc from GlcNAc via GlcNAc 6-P, *N*-acetylmannosamine (ManNAc) 6-P, and ManNAc by the use of *E. coli* cells producing GlcNAc 6-P 2-epimerase and NeuAc synthetase, in expectation of biological functions of *E. coli* such as the supply of phosphoenolpyruvate (PEP), which is an essential substrate for NeuAc synthetase, GlcNAc phosphorylation by the PEP-dependent phosphotransferase system, and dephosphorylation of ManNAc 6-P. Eleven mM NeuAc was synthesized from 50 mM GlcNAc by recombinant *E. coli* cells with the addition of glucose as an energy source. Next we attempted to synthesize CMP-NeuAc from GlcNAc and CMP using yeast cells, recombinant *E. coli* cells, and *H. influenzae* CMP-NeuAc synthetase, and succeeded in efficient production of CMP-NeuAc due to a sufficient supply of PEP and efficient conversion of CMP to cytidine 5'-triphosphate by yeast cells.

Key words: *N*-acetylneuraminic acid; cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc); *N*-acetylglucosamine (GlcNAc) 6-P 2-epimerase; NeuAc synthetase

A major material of the sialic acid group, *N*-acetylneuraminic acid (NeuAc), is incorporated at the terminal of the oligosaccharide chain of glycoconjugates, and plays important roles in cellular recognition processes and the binding of toxins and viruses.^{1,2)} Hence sialyloligosaccharides and their derivatives have been expected to be utilized as pharmaceuticals and biologically functional materials.

Sialyloligosaccharides can be synthesized chemi-

cally or enzymatically. In the case of chemical synthesis, complicated protection and deprotection steps are required due to complexity in the control of both the stereo- and the regiochemistry of bond formation. On the other hand, enzymatical synthesis can be done stereospecifically with sialyltransferases, which require the active form of NeuAc, cytidine 5'-monophospho-*N*-acetylneuraminic acid (CMP-NeuAc), as a donor substrate.³⁾ Recently many kinds of sialyltransferase genes have been cloned and expressed from mammalian and bacteria cells, and several sialyltransferases are available for the synthesis of sialyloligosaccharides.⁴⁻⁷⁾ But not only the substrate CMP-NeuAc, but also NeuAc is expensive and not readily prepared in large quantities, which prevents the practical production of sialyloligosaccharides by enzymatical methods.

NeuAc has been prepared from natural sources such as colominic acid,⁸⁾ milk, and eggs.⁹⁾ Although enzymatic synthesis of NeuAc from *N*-acetyl-D-mannosamine (ManNAc) using NeuAc lyase (EC4.1.3.3) has been studied,¹⁰⁻¹²⁾ this method is not used because of the expense and the commercial unavailability of ManNAc. Maru *et al.* have reported the production of NeuAc from GlcNAc and pyruvate using porcine *N*-acetyl-D-glucosamine 2-epimerase and *E. coli* NeuAc lyase,¹³⁾ but there still is a problem in that the NeuAc lyase reaction requires a large amount of pyruvate. Recently Tabata *et al.* established a promising method for production of NeuAc by coupling bacteria expressing *N*-acetyl-D-glucosamine 2-epimerase and NeuAc synthetase without the use of pyruvate.¹⁴⁾

CMP-NeuAc is enzymatically synthesized from CTP and NeuAc by the action of CMP-NeuAc synthetase (EC.2.7.7.43).¹⁵⁻¹⁷⁾ Simon *et al.* reported the synthesis of CMP-NeuAc from GlcNAc and CMP as starting materials.¹⁸⁾ But their method is not available for large scale production of CMP-NeuAc because it entails complicated steps such as epimerization of GlcNAc under alkaline conditions, condensation of ManNAc and pyruvate to obtain NeuAc, and generation of CTP using the expensive ATP regeneration system. Recently Endo *et al.* also reported a CMP-NeuAc production system from orotic acid and NeuAc by bacterial coupling.¹⁹⁾

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Abbreviations: CMP, cytidine 5'-monophosphate; CMP-NeuAc, cytidine 5'-monophospho-*N*-acetylneuraminic acid; CTP, cytidine 5'-triphosphate; NeuAc, *N*-acetyl-D-neuraminic acid; PEP, phosphoenolpyruvate

We have succeeded in large scale production of several sugar nucleotides by coupling yeast cells and enzymes involved in the metabolism of sugar nucleotides.²⁰⁾ Yeast cells have strong activity of nucleotide-phosphorylation yielding nucleoside 5'-triphosphates from nucleoside 5'-monophosphates, and strong pyrophosphatase activity that enhances the sugar nucleotide synthesis reaction reversibly catalyzed by sugar nucleotide metabolizing enzymes. To establish a practical method for production of CMP-NeuAc from inexpensive materials, GlcNAc and CMP, as primary substrates, we attempted to construct recombinant *E. coli* cells that can convert GlcNAc to NeuAc, and to combine the recombinant *E. coli* cells and yeast cells in the presence of CMP-NeuAc synthetase to produce CMP-NeuAc.

Materials and Methods

Bacteria, plasmid, and culture. *E. coli* JM109²¹⁾ and DH1²²⁾ were used as host cells for cloning and expression of the *neuB1*, *nanE*, and *neuA* genes. Two times YT medium (2 × YT medium)²¹⁾ was used for cultivation of *E. coli* cells. Plasmid pTrc99A²³⁾ carrying the *trc* promoter was purchased from Amersham Bioscience (Piscataway, NJ).

Recombinant DNA techniques. Chromosomal DNA of *Haemophilus influenzae* (ATCC9745) and *Campylobacter jejuni* strain 1652 were obtained from Mr. N. Matsumoto and Dr. T. Shiba of the Graduate School of Technology of Hokkaido University. Isolation of plasmid, digestion of DNA with restriction enzymes, ligation, transformation, and agarose gel electrophoresis were done in the usual ways.²⁴⁾

Construction of expression plasmids. *H. influenzae* DNA containing the coding region of *nanE* (HI0145)²⁵⁾ was amplified as a *XbaI*–*SalI* fragment by the PCR method using two primers: 5'-GGTCTAGATTTAAATGAGGGGTGTTATATGT-3' and 5'-TCGTCGACTTATCTTGCAGATTTCACTGAATTAGCAAACCA-3'. A 720 b DNA fragment was cloned into the *XbaI* and *SalI* sites in pTrc99A. The constructed plasmid was designated pTrcnanE.

The coding region of the *nanK* gene²⁵⁾ of *H. influenzae* was amplified as a *PstI*–*HindIII* fragment by the PCR method using two primers: 5'-AACTGCAGTTTTGTGATGGCTGAGGGGCGTTATAAC-3' and 5'-CC-AAGCTTATTTTATACGTTGTTCAAGAATTACAT-3'. A 1.8-kb DNA fragment was cloned into the *PstI* and *HindIII* sites in pTrc99A, yielding pTrcnanKR.

The coding region of *neuB1* of *Campylobacter jejuni* genome²⁶⁾ was amplified by the PCR method with two primers: 5'-TACGATTATTTTCCTGATGCTC-3' and 5'-TCTCCAAGCATTAACGCC-3'. The *neuB1* coding region was amplified as a *BamHI* and *PstI* fragment by the nest PCR method using two primers: 5'-AAGGATCCTCTGGTGAGGCTTATGGAA-3' and 5'-GTCT-

GCAGATTTAATCTTAGAATAATCAGCCC-3'. A 1.2-kb DNA fragment was cloned into the *BamHI* and *PstI* sites in pTrc99A. The resulting plasmid was designated pTrc-neuB1.

After pTrc-NanE was cleaved with *SalI* and filled in with T4 DNA polymerase, the *SalI*–*PstI* fragment containing the *nanE* gene was excised and inserted into the *BamHI*–*PstI* sites in pTrc-neuB1, whose *BamHI* site had been cleaved with *BamHI* and then filled in with T4 DNA polymerase. The resulting plasmid containing the *nanE*–*neuB1* genes under the *trc* promoter was designated pTrc-NENB.

Enzyme preparation. Transformed *E. coli* cells were basically cultivated in 2 × YT medium with 100 µg/ml of ampicillin and 1 mM manganese chloride at 37 °C to 5 × 10⁸ cells/ml, and then isopropyl-β-D-thiogalactopyranoside (IPTG, final 0.2 mM) was added to the culture. After 8 h of induction, cells were harvested by centrifugation and suspended in a buffer of 20 mM potassium phosphate (pH 8.0). Cells were disrupted by sonic oscillation and cell-free extract was prepared by centrifugation (15,000 × g, 4 °C, 10 min). *E. coli* cells possessing pTrc-NENB, used for NeuAc and CMP-NeuAc synthesis, were cultivated in the same way. *H. influenzae* CMP-NeuAc synthetase was prepared as described previously.²⁷⁾

Enzyme assay. NeuAc synthetase (NeuB1) activity was measured as follows: The assay mixture contained 100 mM Tris–HCl (pH 7.0), 20 mM ManNAc, 25 mM phosphoenolpyruvate (PEP), 10 mM manganese chloride, and the enzyme, and was incubated at 37 °C for 5 min. Then the reaction was stopped by heating at 100 °C for 3 min, followed by rapid cooling. Conversion of ManNAc to NeuAc was measured by high-performance anion-exchange chromatography pulsed amperometric detection (HPAE-PAD).²⁸⁾ One unit of enzyme was defined as the amount of enzyme forming 1 µmole of NeuAc per min under these conditions.

The GlcNAc 6-P 2-epimerase activity of NanE was measured as follows: The reaction mixture, containing 50 mM Tris–HCl (pH 8.0), 10 mM ManNAc, 20 mM ATP, 20 mM magnesium chloride, 1 unit/ml NanK, and the enzyme, was incubated at 37 °C for 5 min. Then it was heated in a boiling water bath for 3 min, followed by the addition of 1/10 volume of 1 M Tris–HCl (pH 9.0) and 1 unit of alkaline phosphatase. After incubation at 50 °C for 30 min, the amount of GlcNAc produced was measured by HPAE-PAD. One unit of enzyme was defined as the amount of enzyme forming 1 µmole of GlcNAc(6-P) per min under these conditions.

The ManNAc kinase activity of NanK was measured as follows: The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 10 mM ManNAc, 15 mM ATP, 20 mM magnesium chloride, and the enzyme, and was incubated at 37 °C for 5 min. Then it was heated in a boiling water bath for 3 min followed by rapid cooling. The decrease

in ManNAc was measured by HPAE-PAD. One unit of enzyme was defined as the amount of enzyme consuming 1 μ mole of ManNAc per min under these conditions.

CMP-NeuAc synthetase (NeuA) activity was measured by the method described previously.²⁷⁾

HPAE-PAD analytical procedure. High-performance anion-exchange pulsed amperometric detection (HPAE-PAD) was done on a Dionex DX-500 system (Dionex, Sunnyvale, CA) equipped with CarboPac PA1 column (4 \times 250 mm). A gradient was used to separate the components studied; Method-1: T₀ = 0.1 N NaOH, 0.09 M Na-acetate; T₁₀ = 0.1 N NaOH, 0.09 M Na-acetate; T₁₅ = 0.1 N NaOH, 0.25 M Na-acetate; T_{15.01} = 0.1 N NaOH, 0.5 M Na-acetate; T₂₀ = 0.1 N NaOH, 0.5 M Na-acetate for NeuAc analysis. Method-2: T₀ = 0.018 N NaOH; T₂₀ = 0.018 N NaOH; T_{20.01} = 0.1 N NaOH, 0.5 M Na-acetate; T₂₇ = 0.1 N NaOH, 0.5 M Na-acetate for ManNAc and GlcNAc analysis.

HPLC analysis. The conditions for HPLC analysis were as follows: column, YMC HS-302 (4.6 \times 150 mm); detection, 270 nm; mobile phase, 0.2 M triethylamine-phosphate (pH 6.0); column temperature, 22 °C; flow rate, 0.6 ml/min.

NeuAc synthesis. The reaction for NeuAc production was basically done in a test-tube (2.2 \times 20 cm). The reaction mixture consisted of 75 mM potassium phosphate buffer (pH 8.0), 50 mM GlcNAc, 30 mM magnesium chloride, 200 mM glucose, 0.5% (v/v) xylene, and *E. coli* cells possessing pTrc-NENB recovered from 25 ml culture. The reaction volume was adjusted to 5 ml with distilled water. The reaction mixture was incubated at 28 °C with stirring. One hundred eighty mg of glucose was added to the reaction mixture after 9 and 23 h. The amount of NeuAc synthesized was measured by HPAE-PAD.

CMP-NeuAc synthesis. The CMP-NeuAc synthesis reaction mixture consisted of 200 mM potassium phosphate buffer (pH 8.0), 100 mM GlcNAc, 50 mM CMP, 30 mM magnesium chloride, 200 mM glucose, 0.5% (v/v) xylene, 8.5 units of CMP-NeuAc synthetase, 4.0% (w/v) baker's yeast, and *E. coli* cells possessing pTrc-NENB from 25 ml culture. The reaction volume was adjusted to 5 ml with distilled water. The reaction was incubated at 28 °C with stirring. One hundred eighty mg of glucose was added to the reaction mixture after 9, 23, and 32 h. The amounts of CMP-NeuAc and other relative compounds were measured by HPLC.

Protein assay. Protein was measured with the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard.

Chemicals and other materials. Reaction enzymes, T4 DNA ligase, and T4 DNA polymerase were purchased

from Takara Bio (Ohtsu, Japan). GlcNAc, ManNAc, and NeuAc were purchased from Nacalai Tesqu (Kyoto, Japan). Baker's yeast (Oriental Dry Yeast) and alkaline phosphatase from calf intestine (CIAP) were purchased from the Oriental Yeast (Osaka, Japan). Nucleotides, nucleosides, and bases were prepared at the Yamasa Corporation (Choshi, Japan).

Results

Strategy for production of CMP-NeuAc

In bacteria cells, NeuAc is generally incorporated into the cell by NanT (NeuAc transporter), and reversibly cleaved by NanA (NeuAc lyase), generating ManNAc and pyruvate, and ManNAc is then phosphorylated by NanK (ManNAc kinase), yielding ManNAc 6-phosphate (ManNAc 6-P), as shown in Fig. 1. On the other hand, ManNAc and GlcNAc are incorporated as phosphorylated sugars (ManNAc 6-P and GlcNAc 6-P) by transporters (ManXYZ and NagE).^{29,30)} ManNAc 6-P is then converted to GlcNAc 6-P by NanE (GlcNAc 6-P 2-epimerase). The resulting GlcNAc 6-P is metabolized by NagA (GlcNAc 6-P deacetylase) and NagB (isomerizing GlcNAc 6-P deaminase). Thus NeuAc can be synthe-

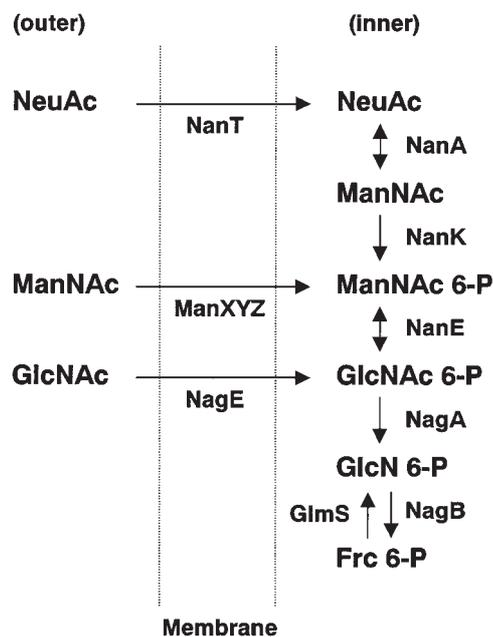


Fig. 1. Metabolism of NeuAc in Bacteria Cells.

NeuAc is taken up as a free sugar by NanT and is cleaved by NeuAc lyase (NanA) to give ManNAc and pyruvate. ManNAc is phosphorylated by NanK, producing ManNAc 6-P. ManNAc 6-P is converted to GlcNAc 6-P by NanE, the substrate of NagA. Fru 6-P converted from GlcN 6-P by NagB is metabolized through the glycolysis pathway. GlcNAc and ManNAc are also metabolized after they are taken up as phosphorylated sugars by transporters. NanT, NeuAc transporter; NanA, NeuAc lyase; NanK, ManNAc kinase; NanE, GlcNAc 6-P 2-epimerase; NagA, GlcNAc 6-P deacetylase; NagB, isomerizing GlcN 6-P deaminase; GlimS, GlcN 6-P synthase; ManXYZ, ManNAc transporter; NagE, GlcNAc transporter.

sized from GlcNAc by the actions of NagE, NanE, NanA, and the dephosphorylation activity of ManNAc 6-P. Since NanA prefers the NeuAc-degradation reaction to the NeuAc-synthesis reaction (condensation of ManNAc and pyruvate), the addition of excess amounts of pyruvate is essential to cause NanA to catalyze the NeuAc-synthesis reaction. To avoid the use of excess pyruvate, we tried to utilize bacterial NeuAc synthetase (NeuB) derived from *Campylobacter jejuni*, which irreversibly catalyzes the synthesis of NeuAc using phosphoenol pyruvate (PEP). First we attempted to construct recombinant *E. coli* cells which produce both *Haemophilus influenzae* NanE and *Campylobacter jejuni* NeuB in order to synthesize NeuAc from GlcNAc, anticipating *E. coli* biological functions such as the supply of PEP, an essential substrate for NeuAc synthetase, GlcNAc phosphorylation by the PEP-dependent phosphotransferase system (PTS), and dephosphorylation of ManNAc 6-P, as shown in Fig. 2. To provide the substrate CTP for CMP-NeuAc synthesis, a combination of Baker's yeast cells and CMP was used, in expectation of a sufficient supply of PEP through glycolysis in yeast cells. Coupling of the recombinant *E. coli* cells producing NanE and NeuB, Baker's yeast cells, and CMP-NeuAc synthetase was expected to result in efficient production of CMP-NeuAc using inexpensive GlcNAc and CMP as primary substrates (see Fig. 2).

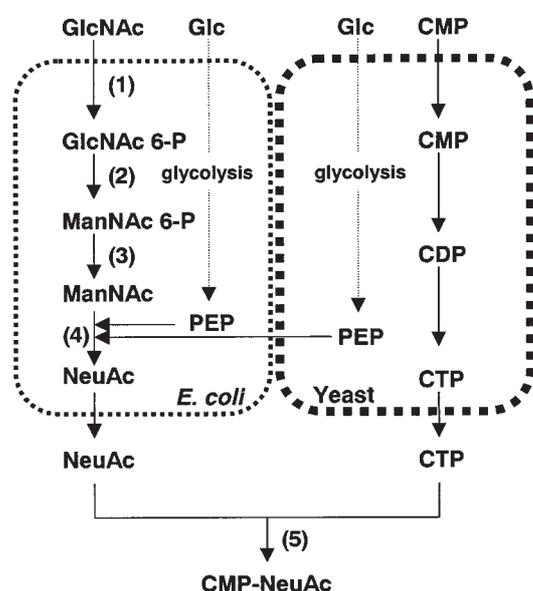


Fig. 2. Scheme for Enzymatic Synthesis of NeuAc and CMP-NeuAc.

NeuAc is synthesized from GlcNAc as a primary substrate by the PEP-dependent phosphotransport system (PTS) (1), GlcNAc 6-P 2-epimerase (NanE) (2), phosphatase (3), and NeuAc synthetase (NanA) (4) in recombinant *E. coli* cells co-producing NanE and NanA proteins. PEP is provided by *E. coli* and yeast cells through glycolysis using glucose (Glc). CMP is converted to CTP by yeast cells, and CMP-NeuAc is synthesized from NeuAc and CTP by CMP-NeuAc synthetase (5).

Cloning and expression of NanE and NeuB genes

Plumbridge and Vimr³¹⁾ reported that cell-free extracts from *E. coli* cells expressing the *yhiJ* gene, which exists in the *nanAT* operon of *E. coli*, showed increased activity converting GlcNAc 6-P to ManNAc 6-P, suggesting that the *yhcJ* gene encodes GlcNAc 6-P 2-epimerase. They renamed the gene *nanE*. In *H. influenzae* genome *nanE* gene homolog HI0145 was found in the putative *nan* operon. To confirm that the HI0145 gene encodes GlcNAc 6-P 2-epimerase, we cloned the coding region of HI0145 and expressed it in *E. coli* cells. Cell-free extracts prepared from JM109 cells carrying pTrc-*nanE* cultivated with IPTG had a high level of GlcNAc 6-P 2-epimerase activity, while that from control cells had no detectable activity (data not shown). These results indicate that HI0145 encodes the GlcNAc 6-P 2-epimerase of *H. influenzae*.

We also tried to clone the *neuB1* gene encoding NeuAc synthetase³²⁾ from *Campylobacter jejuni*. pTrc-*neuB1* plasmid was constructed and introduced into *E. coli* JM109 cells. Cell-free extracts prepared from JM109 cells carrying pTrc-*neuB1* cultured with IPTG had high NeuAc synthetase activity.

Next we constructed the plasmid pTrc-NENB for co-production of NanE and NeuB1 proteins, and introduced it into *E. coli* JM109 cells. The recombinant *E. coli* JM109 cells simultaneously produced the objective gene products as active forms (Table 1).

NeuAc synthesis from GlcNAc with recombinant *E. coli* cells

NeuAc synthesis was first examined using *E. coli* JM109 cells carrying pTrc-NENB cultivated with IPTG to produce *nanE* and *neuB1* gene products. The primary substrate GlcNAc was added to the cells whose cell membranes had been partially destroyed by treatment with xylene. GlcNAc should be phosphorylated to GlcNAc 6-P by *E. coli* PTS followed by conversion of GlcNAc 6-P to ManNAc 6-P by NanE, and ManNAc 6-P should be dephosphorylated by intracellular phosphatase activity. Collected cells were incubated with 50 mM GlcNAc, 50 mM Glucose, and 150 mM pyruvate with vigorous aeration. After 38 h of reaction, only 1 mM NeuAc was synthesized. Since the viability of JM109 cells is less than that of the other *E. coli* strains

Table 1. GlcNAc 6-P Epimerase and NeuAc Synthetase Activities of Cell Free Extract from *E. coli* Transformants

Strain	Activity (units*/mg protein)	
	NanE	NeuB1
JM109/pTrc99A	<0.10	<0.10
JM109/pTrc-NENB	1.32	1.20
DH1/pTrc99A	<0.10	<0.10
DH1/pTrc-NENB	1.49	2.15

*One unit of NanE and of NeuB1 was defined as the amount of enzyme forming 1 μmole of GlcNAc 6-P and NeuAc, respectively under the conditions described in "Materials and Methods".

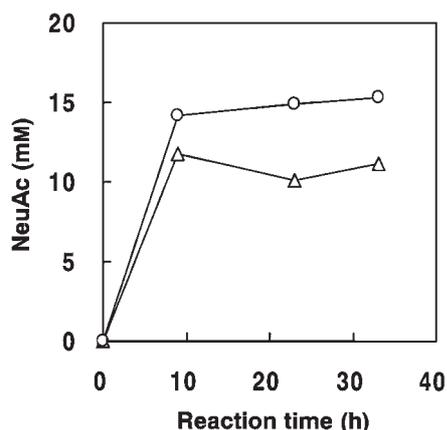


Fig. 3. NeuAc Synthesis by *E. coli* DH1/pTrc-NENB Cells.

NeuAc synthesis from GlcNAc was done with recombinant *E. coli* DH1 cells producing NanE and NeuB1 proteins, as described in "Materials and Methods". The amount of NeuAc was measured by HPAE-PAD. Symbols: open circles, NeuAc formed with 100 mM pyruvate; open triangles, NeuAc formed without pyruvate.

generally used, it was speculated that the supply of PEP and immanent enzyme activities involved in GlcNAc phosphorylation and ManNAc 6-P dephosphorylation were not sufficient, rather than the amount of recombinant enzymes (NanE and NeuB) in the case of the use of JM109 cell as host cells. Hence we screened other several *E. coli* strain transformants for adequate strains for NeuAc synthesis, analyzing abilities to synthesize NeuAc from GlcNAc, and found that DH1 transformant was suitable for NeuAc production. Both the NanE and the NeuB1 enzyme activities of cell-free extract from DH1 carrying pTrc-NENB cells are shown in Table 1.

Using *E. coli* DH1 transformed cells, after 32 h of reaction, 11 mM of NeuAc accumulated from 50 mM of GlcNAc with glucose as the energy source. On the other hand, 15 mM of NeuAc was synthesized after 32 h of reaction when 100 mM pyruvate, a precursor of PEP, was added to the initial reaction mixture, possibly due to the enhanced supply of PEP (see Fig. 3).

CMP-NeuAc synthesis by a combination of Baker's yeast and recombinant E. coli DH1 cells

It was thought that the supply of PEP through the glycolysis in *E. coli* cells was not great enough for the optimal synthesis of NeuAc, as described above. But we expected that Baker's yeast cells would provide not only the energy for the conversion of CMP to CTP, but also a sufficient amount of PEP, due to their strong glycolysis activity. Finally we examined the synthesis of CMP-NeuAc from GlcNAc and CMP by combining the recombinant *E. coli* cells and Baker's yeast cells, and adding the *H. influenzae* CMP-NeuAc synthetase prepared from *E. coli* carrying pTrc-siaB.²⁷⁾ As shown in Fig. 4, after 32 h of reaction, 34 mM of CMP-NeuAc accumulated from 100 mM of GlcNAc and 50 mM CMP. We also attempted to synthesize CMP-NeuAc by a coupling of recombinant *E. coli* and yeast cells by

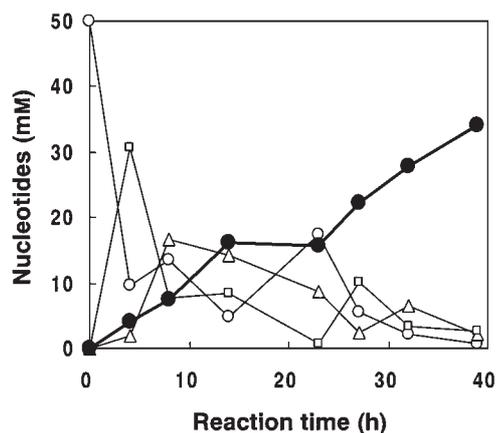


Fig. 4. CMP-NeuAc Synthesis by Coupling Recombinant *E. coli* Carrying pTrc-NENB and Yeast Cells.

CMP-NeuAc was synthesized from GlcNAc, CMP, and glucose by coupling *E. coli* DH1 cells carrying pTrc-NENB and yeast cells in the presence of CMP-NeuAc synthetase. CMP (open circles) was phosphorylated to CDP (open triangles) and CTP (open squares) by yeast cells, and then CTP was converted to CMP-NeuAc (closed circles) by CMP-NeuAc synthetase using NeuAc generated from GlcNAc by recombinant *E. coli* cells.

adding 100 mM pyruvate, but little enhancement of CMP-NeuAc synthesis was observed, suggesting that the supply of PEP by yeast cells was sufficient (data not shown). We did a liter-scale synthesis reaction, and succeeded in 30 mM accumulation of CMP-NeuAc. We confirmed that our method is available for practical production of CMP-NeuAc.

Discussion

Large-scale production of CMP-NeuAc has been greatly desired for enzymatic synthesis of sialyloligosaccharides, but CMP-NeuAc could not be supplied in large quantities at low prices because the substrates, NeuAc and CTP, for enzymatic synthesis of CMP-NeuAc are very expensive and not commercially available in large quantities. To solve these problems, Ichikawa *et al.*³³⁾ developed an *in situ* regeneration system from CMP and NeuAc, and Endo *et al.*¹⁹⁾ also established a production system from orotic acid and NeuAc using bacterial coupling. Although both production systems appeared to be profitable, they still require NeuAc as a primary material.

At first we attempted to use NanA (NeuAc lyase) and NanE (GlcNAc-6P 2-epimerase) of *H. influenzae* to synthesize NeuAc from GlcNAc, and examined NeuAc synthesis with *E. coli* cells producing both enzymes. A significant amount of NeuAc was accumulated only when an excess amount of pyruvate was added, because the equilibrium on NeuAc lyase leans to degradation of NeuAc. Additionally, excess pyruvate inhibited the conversion of CMP to CTP by yeast cells, resulting in inefficient production of CMP-NeuAc in the coupled reaction using recombinant *E. coli* and yeast cells (data

not shown). Hence we decided to use NeuAc synthetase instead of NeuAc lyase because the former catalyzes irreversible condensation of ManNAc and PEP to give NeuAc.

Using *E. coli* DH1 cells producing *C. jejuni* NeuB1 and *H. influenzae* NanE, 11 mM NeuAc was synthesized by adding glucose as an energy source, although only 1 mM NeuAc synthesis was observed in case of the use of JM109 as a host cell. We analyzed the synthesis of NeuAc from GlcNAc using several kinds of *E. coli* strains transformed with pTrc-NENB, and found that DH1 was a suitable host cell for NeuAc synthesis among the tested *E. coli* strains. During screening we did not discover any correlation between the productivities of the two enzymes (NanE and NeuB) and NeuAc synthesis ability. Since this NeuAc synthesis reaction depends not only on two enzyme activities but also on the PEP supply and host *E. coli* enzyme activities involved in GlcNAc phosphorylation and ManNAc 6-P dephosphorylation, we speculated that cell viability, including the ability to effect PEP synthesis, might have more effect than the two enzyme productivities in NeuAc synthesis.

Next we constructed *E. coli* DH1 cells producing not only NanE and NeuB1 but also *Bacillus subtilis* glucokinase,²⁰⁾ which can phosphorylate GlcNAc to give GlcNAc 6-P, because we were afraid that xylene-treatment of *E. coli* cells might lead to inactivation of PTS, and that phosphorylation of GlcNAc might be a limiting factor in NeuAc synthesis. But no enhancement of NeuAc synthesis was observed with the addition of the GlcNAc phosphorylation enzyme, suggesting that PTS should be active even though the cell membrane had been destroyed by xylene (data not shown).

When pyruvate was additionally supplied to recombinant DH1 cells, NeuAc synthesis was significantly enhanced, suggesting that the intracellular level of PEP in *E. coli* cells was a limiting factor in NeuAc synthesis, and that pyruvate served not only as an energy source but also as a precursor of PEP. In addition, we examined the effect of overexpression of the *E. coli* *pckA* gene encoding PEP carboxykinase that synthesizes PEP from oxaloacetate and ATP.³⁴⁾ By the use of *E. coli* cells producing NeuB1, NanE, and PckA, synthesis of NeuAc was slightly enhanced (data not shown).

Baker's yeast cells have been used for production of sugar nucleotides because of their strong ability to phosphorylate nucleoside 5'-mononucleotides, yielding nucleoside 5'-triphosphates using biological energy generated through glycolysis. Hence it was expected that not only ATP but also PEP would efficiently be supplied by yeast cells. Finally we tried to synthesize CMP-NeuAc using the recombinant *E. coli* and yeast cells from GlcNAc and CMP, and succeeded in the production of 34 mM CMP-NeuAc.

Tabata *et al.* have reported that NeuAc was efficiently synthesized from GlcNAc by bacterial NeuAc synthetase and *N*-acetyl-D-glucosamine 2-epimerase without

using pyruvate and PEP.¹⁴⁾ The use of *N*-acetyl-D-glucosamine 2-epimerase is promising for practical production of NeuAc due to the absence of a requirement for phosphorylation of GlcNAc and dephosphorylation of ManNAc 6-P, which can save biological energy as compared to our method. We would like next to attempt to utilize the *N*-acetyl-D-glucosamine 2-epimerase instead of GlcNAc 6-P 2-epimerase to improve our method of CMP-NeuAc production.

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